

## Serum Antibodies to Lipophilin B Detected in Late Stage Breast Cancer Patients<sup>1</sup>

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### ABSTRACT

Lipophilin B mRNA is overexpressed in ~70% of breast tumors and shows a high degree of correlation with the mRNA expression profile of mammaglobin. This is further supported by the recent finding that, like other members of the secretoglobulin-uteroglobin family, mammaglobin and lipophilin B form a heteroduplex. The studies described show that there are pre-existing antibodies to lipophilin B peptide in the sera of breast cancer patients with different stages and grade of tumor and that this response is different from that seen to recombinant mammaglobin and native mammaglobin-lipophilin B complex. The highest titers were observed in later stage tumors. In addition, low levels of antibody were also seen in some patients with prostate and ovarian cancers, consistent with lipophilin B mRNA expression in these tumors at lower abundance than in breast tumors. In contrast, lipophilin B antibodies were absent in 20 healthy donor sera and 30 lung cancer sera. A polymorphism identified in Lipophilin B did not appear to influence human sera reactivity. The data indicate that humoral immune responses to lipophilin B may serve as a diagnostic indicator, particularly for breast cancer.

### INTRODUCTION

Humoral immunity to breast cancer antigens has been studied by several groups in an attempt to relate the presence of pre-existing antibodies to a possible outcome of disease progression or to relate to the original antigen status of the tumor,

thus providing direction for therapeutic targeting. Antibodies have been observed to HER-2/*neu* in sera from patients with breast tumors, particularly in early stage tumors. The incidence of such antibodies is higher in cases of tumors that were identified at the time of diagnosis as having HER-2/*neu* present in the tumor *versus* those not expressing this antigen (1-6). Such antibodies have also been identified in sera of other tumor types (1-3). The presence of HER-2/*neu* in the primary tumor or the presence of antibodies has also played an important role in identifying patients that may be responsive to treatment with Herceptin, a monoclonal antibody specific for HER-2/*neu*. Monitoring for the presence of the extracellular domain of HER-2/*neu* circulating in serum is also used to monitor therapy (5). Antibodies to p53 and a related family member p73 have also been observed in serum of breast cancer patients and are indicative of poor prognosis (7-10), and studies have also demonstrated autoimmunity to MUC-1 (11-14) heat shock proteins (13-15), malignin (16), and Sialyl Tn (17). Serological expression cloning using sera from breast cancer patients and phage-displayed cDNA libraries have also been used to define antigens that participate in the immune response to breast tumors (18-20).

Humoral immunity to a specific antigen in breast cancer can provide prognostic information but can also identify individuals capable of mounting an immune response to that antigen and identifying patients who might benefit from vaccine treatments that target the specific antigen (18-20). Lipophilins and mammaglobins are members of the uteroglobin family, and mammaglobin has been shown to be highly breast tissue specific (21-24). More recently, lipophilin B, which is also present in breast tissue and other tissues, has been identified as forming a complex with mammaglobin that is potentially secreted into serum (25, 26). This study investigates the presence of antibodies to recombinant mammaglobin, native mammaglobin-lipophilin B complex, and lipophilin B in sera from patients with breast cancer at different stages of disease, as well as in other cancers and healthy donor sera to determine the prognostic utility of such antibodies.

### MATERIALS AND METHODS

**Patient Samples.** Sera were obtained from 74 breast cancer patients with various stages of disease progression, as well as from 26 ovarian, 30 endometrial, 39 prostate, and 30 lung cancer patients (Samplex, Westlake Village, CA, and Lifeblood, Memphis, TN).

**Healthy Donor Sera.** Twenty healthy donor sera were obtained from Boston Biomedica, Inc. (West Bridgewater, MA), as well as from volunteer donors at Corixa Corp. (Seattle, WA).

**Peptides.** Mature lipophilin B is a 69 amino acid peptide and was synthesized with Fmoc chemistry using 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate on a Pioneer peptide synthesizer (PE Biosystems, Foster City, CA).

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## 750 Lipophilin B Antibodies

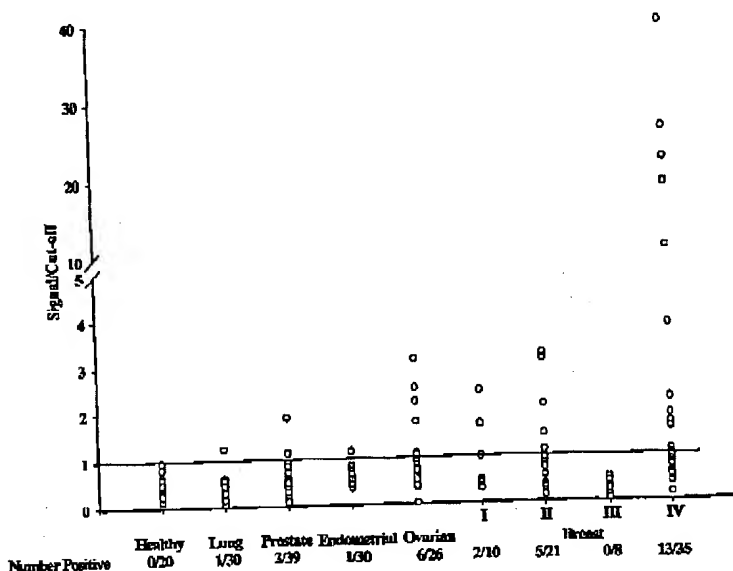


Fig. 1 Antibody responses to lipophilin B in different cancers. Scattergram of reactivity with sera from breast, ovarian, endometrial, prostate, and lung cancer patients and healthy individuals.

The peptide was cleaved from the solid support using standard procedures and purified by reverse phase high-performance liquid chromatography on a C18 column (Vydac), using a gradient of 5–60% acetonitrile (containing 0.05% trifluoroacetic acid) in water (containing 0.05% trifluoroacetic acid). The purified peptide was lyophilized and characterized by matrix-assisted desorption/ionization mass spectrometry before use.

**Recombinant Mammaglobin.** Recombinant mammaglobin was expressed in *Escherichia coli*. cDNA constructs of the full-length mature mammaglobin with NH<sub>2</sub>- and COOH-terminal His-Tags were subcloned into a modified pET 28 vector (27). The construct was then transformed into BL21 pLysS *E. coli* (27) and grown in culture in the presence of kanamycin and chloramphenicol before inducing with isopropyl- $\beta$ -D-thiogalactoside. Recombinant protein was purified with nickel affinity chromatography using an imidazole gradient.

**Mammaglobin/Lipophilin B Complex.** Native mammaglobin/lipophilin B complex was isolated from supernatants obtained from MDA-MB415 cells grown in serum-free media (26).

**EIA.**<sup>3</sup> For detection of human lipophilin B antibody responses, 96-well microtiter plates (Corning Costar, Cambridge, MA) were coated overnight at 4°C with lipophilin B peptide (1  $\mu$ g/well), recombinant mammaglobin (200 ng/well), or purified native mammaglobin/lipophilin B complex (500 ng/well). Plates were then aspirated and blocked with PBS containing 5% nonfat milk for 2 h at room temperature. This was followed by washing in PBST. Serum (1/100)

dilution in PBS containing 5% nonfat dried milk was added to wells and incubated for 2 h at room temperature. This was followed by washing six times with PBST and then incubating with protein A-horseradish peroxidase conjugate at a 1/20,000 dilution in PBST with 0.1% BSA (Sigma Chemical Co., St. Louis, MO) for 1 h. Plates were then washed six times in PBST and incubated with tetramethylbenzidine substrate (Kirkegaard and Perry, Gaithersburg, MD) for 15 min. The reaction was stopped by the addition of 1 N sulfuric acid, and plates were read at 450 nm using an EIA plate reader (Model Elx800; Biotek Instruments, Hyland Park, VA). The cutoff for assays was determined from the mean absorbance of the negative population plus three SDs of the mean. The signal to cutoff was determined from the ratio of the sample EIA absorbance value to the cutoff. To determine titer serum in the assays, we used a dilution of 1/50–1/6,400.

**Quantitative Real-time PCR of Lipophilin B.** Breast tumors, ovarian tumors, and prostate tumors, along with their corresponding normal tissue and other normal tissues, were tested in quantitative (real time) PCR. This was performed on a GeneAmp 5700-sequence detection system (PE Biosystems) using the SYBR green assay system. The primers used for lipophilin B detection were: (a) forward 5'-TGCCCTCCGGAAGCT-3'; and (b) reverse: 5'-CGTTCTGAAGGGA-CATCTGATC-3'. A standard curve is generated for the housekeeping gene  $\beta$ -actin, ranging from 200 to 2000 pg to enable normalization to a constant amount of  $\beta$ -actin. This allows the evaluation of the overexpression levels seen in different tumor types.

**Antibodies.** A mouse (2D3) monoclonal antibody was made to recombinant mammaglobin using conventional methods. A rabbit polyclonal was prepared to lipophilin B, as well as the mammaglobin-lipophilin B complex isolated from MB415 cell line supernatants.

<sup>3</sup> The abbreviations used are: EIA, enzyme immunoassay; PBST, PBS containing 0.1% Tween 20.

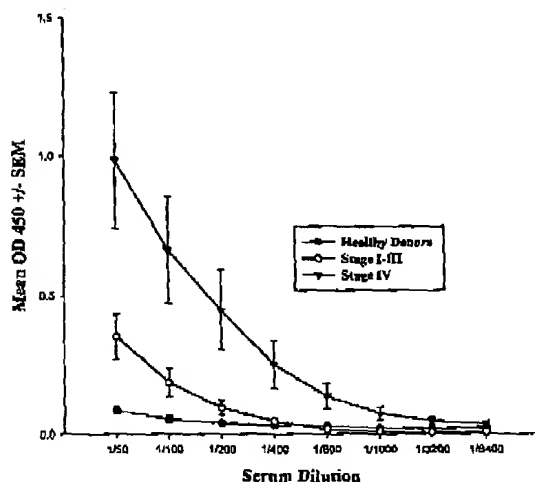


Fig. 2 Titers for lipophilin B antibody-positive sera in stage IV breast tumors ( $n = 13$ ), stage I-III breast tumors ( $n = 7$ ), and healthy donor sera ( $n = 7$ ). The data show the mean EIA absorbance values  $\pm$  SE at different sera dilutions for the individual sera.

Table 1 Breast cancer sera and antibody responses to recombinant mammaglobin and native mammaglobin complex

Sample	Target antigen	
	Mammaglobin	Native complex
Breast tumor stage I	1/10	1/10
Stage II	5/21	2/21
Stage III	0/8	0/8
Stage IV	0/35	8/35
Healthy donors	0/20	0/20

## RESULTS

**Lipophilin B-specific Antibodies in Sera of Breast Cancer Patients.** Studies were performed to determine the presence of antibodies to recombinant mammaglobin, the native complex of mammaglobin and lipophilin B, and free lipophilin B peptide in the sera of breast cancer patients. Differential responses were observed in all three cases. Antibodies to the lipophilin B synthetic peptide were observed in 20 of 74 sera from breast cancer patients. Of the stage IV breast cancer sera tested, 13 of 35 were positive for antibodies to lipophilin B, as shown in Fig. 1, with indications of increased antibody titer in later stage tumors (Fig. 2). The presence of lower titer antibodies in earlier stage tumors is also shown in Figure 1. In contrast, weak immune responses were seen in some sera from breast cancer patients with mammaglobin and native complex but in different sera than those reactive to the synthetic lipophilin B peptide (Table 1).

Higher titer antibodies to lipophilin B were shown to be more prevalent in late stage tumors with 13 of 35 stage IV tumors exhibiting antibodies, some of which had titers of  $>1:1000$ . Fig. 2 shows the titration curve for healthy donor sera

Table 2 Lipophilin B mRNA expression in normal and cancer tissues determined by quantitative PCR\*

Tissue	n	Mean copies/ng $\beta$ -actin	Positive
Breast tumor	25	3393.4 $\pm$ 12724.5	10
Breast normal	4	175.1 $\pm$ 197.4	2
Ovarian tumor	22	275.1 $\pm$ 457.5	12
Ovarian normal	5	27.1 $\pm$ 33.0	0
Prostate tumor	24	98.5 $\pm$ 126.9	7
Prostate normal	4	87.2 $\pm$ 63.3	1
Colon tumor	26	0.37 $\pm$ 0.71	0
Colon normal	7	0.89 $\pm$ 0.79	0
Skin	4	2199.5 $\pm$ 2565.9	3
Skeletal muscle	3	686.7 $\pm$ 913.3	2
Salivary gland	2	2497.5 $\pm$ 3493.8	1
Adrenal	2	129.8 $\pm$ 161.3	1
Retina	2	120.1 $\pm$ 114.5	1
All other tissues	60	14.3 $\pm$ 23.2	2

\* Cut off, mean normals + 3 SD = 83.7.

( $n = 7$ ), stage I-III antibody-positive sera ( $n = 7$ ), and stage IV antibody-positive sera ( $n = 13$ ). This clearly demonstrates the increased titer in sera from stage IV patients as compared with earlier stages of breast cancer.

**Lipophilin B Antibodies in Other Cancers and Normal Donors.** Sera from patients with ovarian, prostate, and lung cancer were also tested for the presence of lipophilin B antibodies. Low titer antibodies were seen in sera from 6 of 26, 1 of 30, and 3 of 39 ovarian, endometrial, and prostate cancer patients, respectively. Of 30 lung cancer patient sera tested, only 1 showed a borderline response (signal/cutoff 1.23). The mean signal to cutoff values for the 6 positive ovarian tumors ranged from 1.03 to 3.1 as compared with 1.07–39.36 for breast cancer sera.

No antibody response to lipophilin B alone, complexed with mammaglobin or to free recombinant mammaglobin, was observed in 20 sera from normal donors, further supporting the specific response to lipophilin B observed in breast cancer patients.

**mRNA Expression in Breast, Ovarian, and Prostate Tumors.** Lipophilin B exhibits an mRNA profile in breast tumors similar to mammaglobin, and although both share some mRNA expression in skin and salivary gland, lipophilin B is also expressed in skeletal muscle, adrenal gland, cartilage, and retina (Table 2). However, unlike mammaglobin that is specific for breast tumors, lipophilin B is also expressed at  $\sim 10$ -fold lower levels in ovarian cancers and prostate cancers. No expression of lipophilin B was detected in colon tumors (Table 2). Both lipophilin B and mammaglobin had similar mRNA expression profiles in a tumor cell line panels comprised of breast, prostate, ovarian, and colon cell lines (data not shown). On this panel, both lipophilin B and mammaglobin had mRNA levels elevated in MDA-MB415 (++++), BT474 (++++), and SKBR-3 (+) cells with relatively similar expression profiles. Lipophilin B mRNA was expressed at a low level in LNCaP prostate cancer-derived cells.

**Polymorphism of Lipophilin B.** Because of the disulfide linkages between mammaglobin and lipophilin B, the analysis of cDNA polymorphisms in lipophilin B was studied to determine their potential impact on antibody responses in breast cancer patients (25, 26).

## 752 Lipophilin B Antibodies

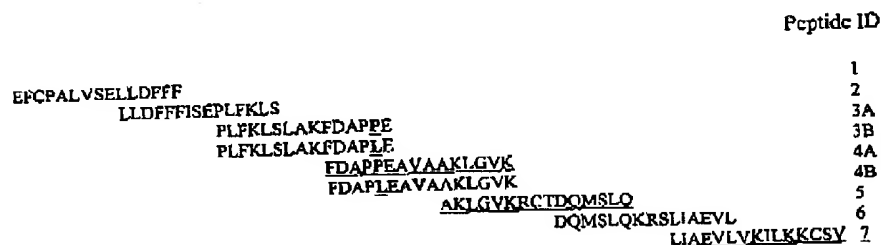
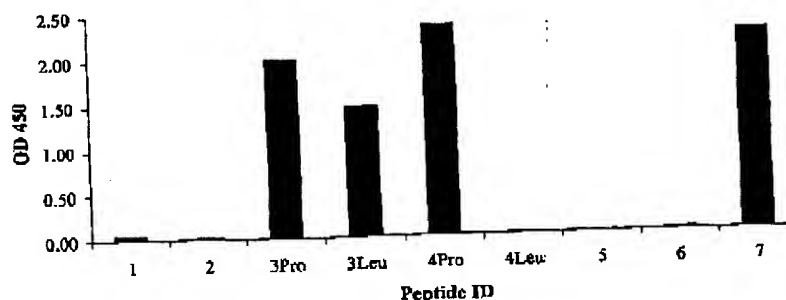


Fig. 3 Sequences of the overlapping synthetic peptides used in the epitope mapping of lipophilin B polymorphs.

Fig. 4 Reactivity of the individual peptides in EIA using rabbit antilipophilin B antibody and human breast cancer sera (stage IV) and normal sera.



The polymorphism study focused primarily on cDNA from primary and metastatic breast tumors. Briefly, the approach used was to create single-stranded cDNA from total RNA isolated from each tumor. This was used as a template for PCR amplification of lipophilin B genes. The tumors used in the analysis had been shown previously to express lipophilin B mRNA by quantitative PCR. Lipophilin B primers were selected outside of the region encoding the open reading frame so that sequence variants within and adjacent to the coding region could be detected. Primers were designed outside the region encoding the open reading frame excepting that the 5' primer included part of the region encoding the signal sequence, attributable to the constraints of the 5' untranslated region sequence on primer design. This enabled sequence variants within and adjacent to the coding region to be detected. Each amplification of lipophilin B was performed with plaque-forming unit polymerase to limit to a minimum the introduction of PCR-induced sequence variants. Each of the PCR amplifications was subcloned, and five independent clones were subjected to DNA sequence analysis. Both strands were sequenced to reduce the possibility of sequencing errors.

Twenty breast tumors (11 metastatic and 9 primary) were used in this study, along with a single normal breast sample. The key findings for the analysis of the lipophilin B sequence variants were that a single prevalent sequence variant resulted in a C-T transition at bp 158, resulting in a proline to leucine change at the amino acid level. Eleven of the 20 tumors analyzed contained some cDNAs with this sequence variant.

**Epitope Mapping of Lipophilin B Relative to Known Polymorphisms.** Several overlapping peptides were synthesized that spanned the full-length lipophilin B sequence (Fig. 3).

This included peptides that represented the proline to leucine change. All of these peptides were tested in EIA with a rabbit polyclonal antisera made to the proline version and human breast cancer sera to pinpoint the epitopes. Rabbit antilipophilin B reacted specifically with the COOH-terminal peptide 7 but was also reactive with peptides in and around the region where the proline to leucine polymorphism occurs with peptides 3A and B being reactive, as well as 4A (Fig. 4). Human sera reactivity, however, appeared to be located in peptide 5 but with less overall reactivity than the full-length lipophilin B peptide (Fig. 5). Such a response should therefore be largely independent of sequence variation occurring at upstream sites (peptides 3A and B). This is consistent with the model. On the basis of the molecular model of the complex (26), the polymorphism is not located near the hydrophobic binding cavity. Rather, it is predicted to be in a loop connecting two  $\alpha$  helices and at the surface of the molecule. The proline in the more abundant form likely assists in creating this loop but is not critical, because there is a second proline adjacent to the polymorphism.

**Antibody Responses to Mammaglobin, Complex, and Lipophilin B.** The human antibody response to the mammaglobin-lipophilin B complex or recombinant mammaglobin was also frequently different from that seen with lipophilin B. In Fig. 6, a monoclonal antibody 2D3 raised to the recombinant mammaglobin only reacted with the recombinant and not to the native complex or lipophilin B. In contrast, the polyclonal anticomplex antibody reacted with both the recombinant mammaglobin and the complex but not lipophilin B. The polyclonal antibody to lipophilin B reacted with the complex, as well as lipophilin B, but not recombinant mammaglobin. In human breast cancer sera, very little antibody response was seen to

Fig. 5 Reactivity of the individual peptides in EIA using human breast cancer sera (stage IV) and normal sera.

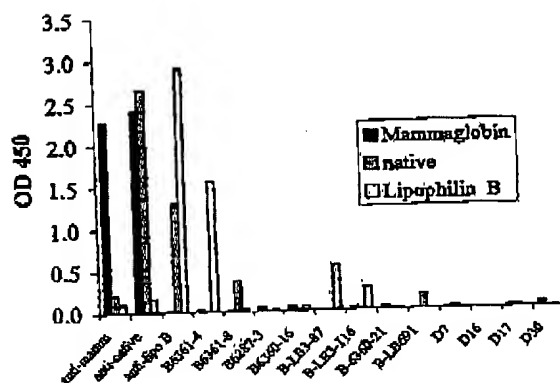
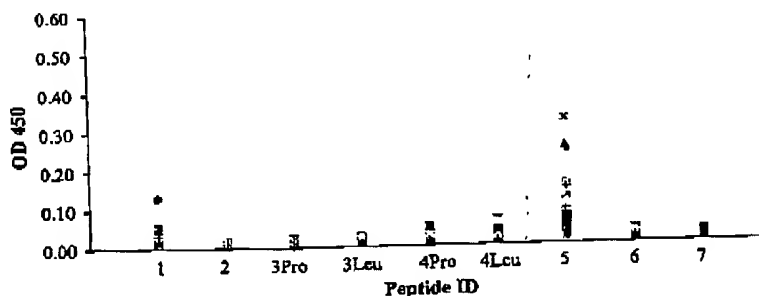


Fig. 6 EIA reactivity of recombinant mammaglobin, native complex, and lipophilin B with antibodies and with human sera from breast cancer patients and normal individuals.

recombinant mammaglobin. The strongest responses were to lipophilin B but also to the complex. However, in many cases, the sera reactive with the complex were not the same as those reacting to lipophilin B, implying different epitopes are involved. Examples of such reactivity are shown in Fig. 6.

## DISCUSSION

EIA detected antibodies specific to lipophilin B in 27% (20 of 74) sera from breast cancer patients. Higher titer antibodies, with in some cases titers  $>1:1000$ , were observed in stage IV tumors (37.1%, 13 of 35). In contrast, only weak responses were observed to mammaglobin, either as a purified recombinant or complexed with lipophilin B. The strong response to lipophilin B and not to the complex would also indicate that lipophilin B may exist in the sera of breast cancer patients in the free form. It may also indicate that in the complex, it is folded so that it is not accessible. Sera that were positive for lipophilin B antibodies should also be different from those expected to exhibit responses to HER-2/neu that tend to predominate in early stage tumors (4). Although some sera were reactive with the native mammaglobin-lipophilin B complex, they tended to be different from those with lipophilin B antibodies, indicating the involvement of different epitopes.

In the case of other cancers, although there were detectable

antibody levels in several sera from ovarian cancer patients, the levels were significantly less than seen in breast cancer patients. Prostate and lung cancer sera had little or no significant antibody responses to lipophilin B (Fig. 1). The detection of low responses to ovarian cancers is consistent with the 10-fold lower mRNA expression levels detectable in ovarian tumors than in breast tumors.

Epitope mapping of the polymorphs of lipophilin B indicated that the predominant human antibody response was to peptide 5 (Fig. 3). This is in contrast to the location of epitopes reacting with a rabbit polyclonal antibody to lipophilin B peptides 3Pro, 3Leu, 4Pro, and 7. Reactivity to peptide 5, however, is much weaker than seen with the complete lipophilin B protein, indicating the possibility of conformational epitopes contributing to the recognition by human sera.

In summary, there is a high degree of correlation between the antibodies to lipophilin B in serum to the presence of breast cancer. Similar antibody responses have been seen to other breast cancer markers, e.g., HER-2/neu. The presence of antibodies to HER-2/neu has been highly indicative of the potential of this protein as a target for immunotherapy, and indeed, it has been targeted for monoclonal antibody therapy and as a vaccine target. Many tumors, however, do not express HER-2/neu, and there is a need to identify other candidate antigens that exhibit humoral responses in cancer patients that may be immunotherapeutic targets. For this purpose, many groups have used serological expression techniques to identify such antigens. Lipophilin B, although eliciting an immune response, is also linked covalently to mammaglobin, a highly glycosylated protein that may complicate its use in immunotherapy. The presence of lipophilin B-specific antibodies in serum may, however, serve as a diagnostic indicator of breast cancer.

## ACKNOWLEDGMENTS

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